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13. ABSTRACT ***(Maximum 200 Words)***
In studies to define the changes in gene expression after expression of telomerase in normal human breast epithelial cells using microarray, we have focused on obtaining appropriate cell strains and lines for use with Affymetrix arrays in order to compare with spotted arrays from commercially available sources. Thus far, although still quite preliminary, our data indicate a relationship between the life span extended cells and the spontaneously immortal cells rather than those cells that are approaching senescence in terms of gene expression patterns. Interestingly, the telomerase-expressing normal mammary cells have similar expression patterns to the spontaneously immortal line for certain genes related to proliferation rather than specific markers related to a cancer-like phenotype. A direct comparison to normal young mammary strains is currently underway, with the hope of defining any differences in young diploid normal cells and those that have an extended life span using ectopic expression of human telomerase. Our hypothesis is that expression of telomerase prevents the genomic instability associated with the immortalization process, which would also prevent the change in gene expression patterns observed for the spontaneously immortal lines as well as those genes expressed as cells approach senescence.

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Introduction

Our current data indicate that telomere shortening is a primary cause of cellular senescence as expression of telomerase in normal cells provides maintenance of telomere lengths and prevention of cellular senescence (Bodnar et al., 1998) without signs of cancer-associated changes (Morales et al., 1999). Our goal is to define the molecular consequences of this exogenous expression of telomerase in mammary epithelial cells and determine the changes in gene expression patterns that could distinguish young diploid cells from those that have an extended life span with telomerase from those that have immortalized. It is important to make a distinction between immortalized cells, which require significant gene expression changes, and our normal cells with telomerase, which, while continuously dividing, are not immortal in the classical sense. Our hypothesis is that ectopic telomerase provides primary mammary cells with genomic stability, preventing changes in gene expression typically associated with progression toward a breast cancer-like state. Therefore, we plan to directly test whether telomerase acts as an aging/tumor suppressor by determining the consequences of exogenous telomerase expression in normal human mammary epithelial (HME) and stromal (HMS) cells.

Body

Objective: To define the changes/differences in gene expression patterns between normal, diploid mammary epithelial cells, both young and approaching senescence, telomerase-expressing mammary cells, and spontaneously immortalized mammary lines.

Human mammary epithelial (HME) cells from a patient with Li Fraumeni syndrome (LFS) were isloated and cultured as previously described (Shay et al., 1995). HME 50-5 cells (a clone derived from LFS HME 50, which will be referred to as "50-5") harbor a germline mutation in a single allele of *p53*. Mortal 50-5 cells were stably infected with a retroviral vector encoding hTERT (50-5-hTERT), the catalytic component of human telomerase. As shown in Figure 1, we have successfully been able to generate 50-5 cell lines for over-expression of telomerase. Cells with telomerase activity (indicated by the 6bp laddering effect observed in lanes 4-12) showed elongated telomeres (right panel, Fig 1), and an extended cellular life span without observable senescence as indicated by staining with the senescence marker, β-galactosidase (not shown).

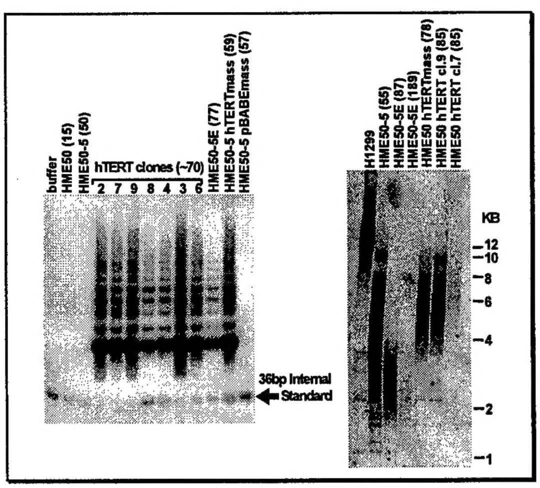

Figure 1. Stable expression of hTERT in HME cells. Left Panel: Stable 50-5 mass cultures and single cell-derived clones ectopically expressing hTERT and telomerase activity. Cultures were obtained by retroviral infection of hTERT, selected, and analyzed for telomerase activity using the TRAP assay. The 36-bp internal standard serves as a control for quantitation of activity. In all cases, 2000 cell equivalents were assayed. Right panel: Maintenance of telomere lengths in stable 50-5 mass cultures and clones expressing exogenous telomerase. hTERT-infected 50-5 cells maintained telomeres at a length of ~6.5kb. The telomeres of the uninfected, parental 50-5 cells were shorter (median TRF of ~4.2kb) and more heterogeneous in length, while 50-5E spontaneously immortalized cells maintained their telomeres at ~2.5kb. H1299, a human lung cancer cell line with unusually long telomeres, was included as a control. Values in parentheses represent the population doubling at the time of isolation.

These 50-5 cell strain is able to spontaneously immortalize in culture with a dramatic increase in aneuploidy (Fig 2) and reactivation of endogenous telomerase (Fig 1, left panel; Shay et al., 1995). Our stable telomerase-expressing 50-5 lines, as with other hTERT-expressing normal cells (Morales et al., 1999), have a stable karyotype and no reactivation of endogenous hTERT (not shown; Elmore et al., 2002).

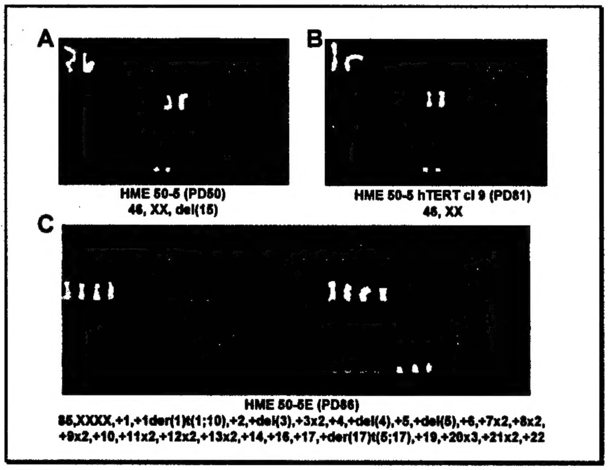

Figure 2. Genomic stability with over-expression of hTERT in HME cells. Representative metaphase spreads are shown following SKY for normal LFS 50-5 (A), hTERT 50-5, (B) and 50-5E (C), the spontaneously immortal line. All of the chromosomes from the exogenous hTERT cells showed a single color (indicating no interchromosomal rearrangements). For the clonal lines expressing exogenous hTERT, the majority of the cells' chromosomal complements were within normal limits, while the 50-5 and the 50-5 E (PD 171) cells had multiple abnormalities (indicated by arrows), which tended to be different in each metaphase spread, demonstrating cytogenetic heterogeneity. (C) shows a representative metaphase spread from the near tetraploid 50-5E line (PD 171) (SKY) that has 15 structurally abnormal chromosomes.

The 3 cell lines represented in Figure 2 have been analyzed for gene expression patterns using the Affymetrix arrays and OpArrays from Operon Technologies, and representative experiments are shown in Figure 3. Importantly, the initial experiment utilized near senescent 50-5, hTERT cl.9, and 50-5E spontaneously immortal line, and these experiments are being repeated with young, diploid, actively dividing 50-5 cells. We have essentially found in our initial data analysis that the hTERT cells and the 50-5E cells are remarkably similar to each other, while the near senescent cells are somewhat dissimilar (Figure 3). Most of the genes involved in the senescence program are related to the IGF-family of binding proteins (IGF-2 binding protein 3, IGF-4 binding protein 4 both down-regulated in near senescent cells compared to hTERT HMEs) and the cathepsin family member proteins, which have associated increased expression of these proteins with aging. In addition, we have found that p57 (kip2) is upregulated as normal cells approach senescence and is absent in the spontenously immortal HME50-5E, suggesting a p53-independent p57 role. More in depth, thorough analysis will be accomplished in the second round of Affymetrix arrays, which will include the comparison of hTERT to young, diploid cells.

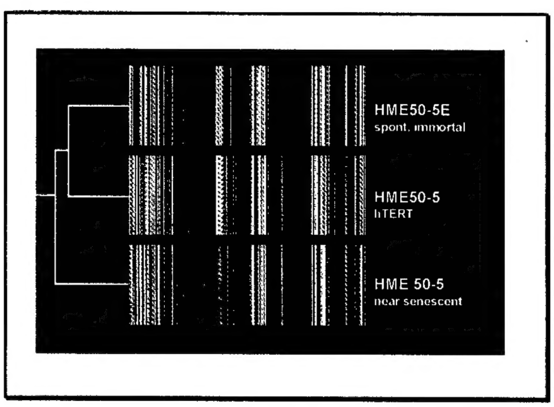

Figure 3. Cluster analysis of HME cell lines reveals similarities between the spontaneously immortal HME50-5E and the HME50-5-hTERT cells. Individual lines in the cluster analysis using the Affymetrix gene chips (HG_U95Av2) revealed that many proliferative genes were commonly expressed in those lines that were actively dividing (HME50-5E and HME50-5-hTERT) compared to the HME cells that were near senescent (HME50-5). More cluster analysis with a second round of hybridizations is forthcoming using young, diploid HME populations.

We have expanded the study to include the differences between breast tumor cells with and without exogenous hTERT after treatment with the chemotherapeutic compound, Adriamycin. Importantly, MCF-7 cells undergo a senescence program after treatment, making the comparison to untreated cells along the lines of normal cells in the proliferative pool versus those approaching senescence. This type of characterization will provide us with important targets related not only to those genes important in adriamycin-induced senescence but also those that may be necessary to replicative senescence observed in normal mammary epithelial cells. Table 1 shows the important cell cycle and p53-related gene expression differences. We are currently following these

Comparative Expression Profiles of Select Targets

	Gene	MCF-7+AdR/ Untreated	MCF-7 neo+AdR/ Untreated	MCF-7 E6+AdR/ Untreated
cell cycle regulators	Cyclin A	10x ↓	3.5x ↓	↑
	Cyclin B	10x ↓	4.8x ↓	↑
	Cyclin E2	10x ↓	absent	↑
	Cyclin F	5.0x ↓	absent	NC(p)
	PCNA	5.0x ↓	2.0x ↓	↑
	cdc-2	6.7x ↓	NC(a)	NC(p)
	TGF-β	14x ↑	26x ↑	1.6x ↓
p53-related	p21 (waf-1)	12x ↑	↑	absent
	GADD45	4.2x ↑	↑	NC(a)
	Bax-α	3.1x ↑	1.9x ↑	absent
	Bax-γ	3.0x ↑	2.3x ↑	absent
	p53	NC(p)	NC(p)	NC(p)
decreased in MCF-7 E6 cells	IGFBP-5	14x ↑	12x ↑	absent
	JAK-1	4.1x ↑	↑	absent
	MRP-3B	↑	3.2x ↑	absent
	c-jun	12x ↑	↑	absent
	BID	4.1x ↑	1.6x ↑	absent
	BIK	↑	↑	absent
	ERF-1	3.2x ↑	11x ↑	6.2x ↓

[confirmation of the following targets was accomplished using the OpArrays from Operon Technologies: cyclins B and E, PCNA, p21, GADD45, c-jun, p53, and superoxide dismutase]

Our goal is not to characterize every gene that is differentially expressed, but to follow important genes related to DNA damage, telomere maintenance, and the senescence program in both the HME model system and the MCF-7 breast tumor cell system. Understanding the role of these genes will provide important data for defining the mechanisms of telomere-related and stress-induced senescence, as well as providing critical preliminary data for future DOD or NIH proposal in breast cancer biology.

Key Research Accomplishments

1-telomerase expressing normal mammary epithelial cells are molecularly distinct from normal mammary cells approaching senescence

2-expression of genes related to proliferation/cell cycle regulation are commonly expressed in immortal mammary cell lines and the matched normal cell strains with etopic telomerase expressed

3-further experiments on intimately comparing the gene expression profiles of young mammary cells with those with telomerase over-expression are underway, but preliminary data suggests few changes in expression patterns

Recommended Changes to the Proposed Work Based on Additional Findings

We have been granted a no-cost extension of the 1-year Concept Award to finish the proposed experiments for reasons related to personnel and the fact that the microarrays from Operon are no longer sold due to licensing and patent problems with Affymetrix, as of July 2001. Because of this, we have had to utilize Affymetrix arrays and directly compare to the few OpArrays we currently have on hand. However, we have access to the newly available arrays from Genospectra (Freemont, CA), which has a limited agreement with Affymetrix in place. In addition, we have expanded our proposed research to looking at breast tumor cell lines with and without telomerase over-expressed (MCF-7 and MCF-7-hTERT) for gene expression changes before and after treatment with either Adriamycin or γ-irradiation, as well as the differences in mammary cells with and without telomerase after treatment. This will provide us with important molecular targets for defining the mechanisms of Adriamycin-induced and γ-IR-induced DNA damage in both tumor and normal cells, which will be useful for future grant applications.

Reportable Outcomes

Manuscripts – none as yet

Abstracts/Presentations

Elmore, L.W., C.I.Dumur, A.Ferreira-Gonzalez, D.A.Gewirtz, and S.E.Holt. AACR: Oncogenomics, Dublin, Ireland, May 2002.

Holt,S.E., C.I.Dumur, A.Ferreira-Gonzalez, D.A.Gewirtz, and L.W.Elmore. AACR: Oncogenomics, Dublin, Ireland, May 2002.

Holt,S.E., C.I.Dumur, A.Ferreira-Gonzalez, D.A.Gewirtz, and L.W.Elmore. DOD Breast Cancer Research Meeting, Orlando, FL September 2002.

Holt,S.E., L.W.Elmore, Y-M.Di, A.Akalin, P.A.McChesney, and D.A.Gewirtz,. DOD Breast Cancer Research Meeting, Orlando, FL September 2002.

Invited Seminars

Holt,S.E. Department of Medicine, University of Colorado Health Science Center, Denver, CO. June 2002.

Holt,S.E. Department of Medical Microbiology and Immunology, Texas A&M Health Science Center, College Station, TX. April 2002.

Holt,S.E. Department of Biochemistry and Genetics, University of Texas Health Center at Tyler, Tyler, TX. April 2002.

Holt,S.E. Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2002.

Holt,S.E. Department of Physiology & Neurobiology, University of Connecticut, Storrs, CT. February 2002.

Holt, S.E. Massey Cancer Center, MCV/VCU. February 2002.

Holt,S.E. Department of Pharmacology/Toxicology, Yale University, New Haven, CT. January 2002.

Development of Cell Lines
We have developed cell lines for telomerase over-expression in HME31, HME50, and MCF-7, and are in the process of making the corresponding mammary stromal cells with telomerase expression for both HME31 and HME50.

Funding Applied For
Department of Defense Breast Cancer Research Program, IDEA award, June 2002

Conclusions
Having established a model system for studying the effects of telomerase over-expression in normal mammary cells, we have utilized Affymetrix microarray technology to analyze the gene expression patterns and how extended lifespan with exogenous telomerase will alter expression. Thus far, this work has identified potential IGF-related and cathepsin family proteins that appear associated with senescence, as well as a single tumor suppressor gene that may also be associated with p53-independent senescence in our Li Fraumeni mammary epithelial cell model system. Our plan is to expand this survey to a more thorough analysis with young diploid cells and breast tumor cells with and without treatment with DNA damaging agents. Our goal is to define changes in gene expression patterns related to telomere-induced or stress-induced senescence in normal mammary and breast tumor cells, as well as determine if long-term expression of telomerase activity in normal mammary cells alters gene expression patterns when compared to normal young HME cells.

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Morales CP, Holt SE, Ouellette M, Kaur K.J, Yan Y, Wilson KS et al. 1999 Absence of cancer associated changes in human fibroblasts immortalized with telomerase. Nat Genet; 21: 115-118.

Shay JW, Tomlinson G, Piatyszek MA, Gollahon LS 1995 Spontaneous *in vitro* immortalization of breast epithelial cells from a patient with Li-Fraumeni syndrome. Mol Cell Biol; 15: 425-432.